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A Single Mutant, A276S of p53 Turns the Switch to Apoptosis

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Abstract

The tumor suppressor protein p53 induces apoptosis, cell cycle arrest, and DNA repair along with other functions in a transcription-dependent manner¹. The selection of these functions depends on sequence-specific recognition of p53 to a target decameric sequence of gene promoters². Amino acid residues in p53 that directly bind to DNA were analyzed, and the replacement of A276 in p53 with selected amino acids elucidated its importance in promoter transcription. For most apoptotic and cell cycle gene promoters, position 9 of the target decameric sequence is a cytosine while for DNA repair gene promoters, thymine is found instead. Therefore, selective binding to the cytosine at the 9th position may transcribe apoptotic gene promoters and thus can induce apoptosis and cell cycle arrest. Molecular modeling with PyMOL indicated that substitution of a hydrophilic residue, A276S, would prefer binding to cytosine at the 9th position of the target decameric sequence whereas substitution of a hydrophobic residue (A276F) would fail to do so. Correspondingly, A276S demonstrated higher transcription of PUMA, PERP, and p21^{WAF1/CIP1} gene promoters containing a cytosine at the 9th position and lower transcription of GADD45 gene promoter containing a thymine at the 9th position compared to wild-type p53. Cell cycle analysis showed that A276S maintained similar G1/G0 phase arrest as wild-type p53. Additionally, A276S induced higher apoptosis than wild-type p53 as measured by DNA segmentation and 7-AAD assay. Since the status of endogenous p53 can influence the activity of the exogenous p53, we examined the activity of A276S in HeLa cells (wild-type endogenous p53) in addition to T47D cells (mutated and mislocalized endogenous p53). The same apoptotic trend in both cell lines suggested A276S can induce cell death regardless of endogenous p53 status. Cell proliferation assay depicted that A276S efficiently reduced the viability of T47D cells more than wild-type p53 over time. We conclude that the predicted preferred binding of A276S to cytosine at the 9th position better transactivates a number of apoptotic gene promoters. Higher induction apoptosis than wild-type p53 makes A276S an attractive candidate for therapy to eradicate cancer.

Keywords

A276S; p53; apoptosis; cell cycle arrest; T47D; tumor suppressor

Introduction

The tumor-suppressor protein p53, also known as “the death star,” is the natural nemesis of cancerous cells^{1,3}. The DNA-binding domain (DBD) binds selectively to double-stranded DNA to execute the destined function of p53. From a cancer therapeutic point of view, cell

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Supporting Information

Additional figure depicts the result from Western Blot experiment. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

cycle arrest and apoptosis are the two most desirable functions of p53 to stagnate and to eradicate cancer cells, respectively⁴. However, it remains unclear how the specific DBD-DNA interaction determines p53's route of action: apoptosis, cell cycle arrest or DNA repair?

The target DNA-binding site for p53 consists of two half-sites with a decameric base pair motif of 5'-RRRCWWGYYY-3' (R=purine, W=A/T, Y=pyridine) separated by 0–13 spacer base pairs^{5,6}. Several crystallographic structures of the p53-DBD complex with various target DNA half-site palindromes were studied to identify the key residues involved in DNA recognition^{7,8}. Seven residues, K120, S241, R248, R273, A276, C277, and R280 are reported present at the p53 DBD-DNA interface that make direct contacts with the DNA half-sites². Therefore, we investigated if any of these direct DNA-binding residues can be altered to improve either apoptosis or cell cycle arrest. S241, R248, and R273 were found to make non-specific contacts with the phosphate backbone of DNA for docking; likewise, R280 anchors non-specifically to the major groove of DNA². This leaves A276, C277, and K120 as critical direct DNA-binding residues that select specific DNA sequences and determine the functional pathway of p53. Of these three, K120 acetylation was found to be crucial for induction of apoptosis but not involved in cell cycle arrest. Correspondingly, the mutation of this residue (the so-called hot-spot mutant K120R) in certain cancers allows these tumors to evade apoptosis⁹. Therefore, sans post-translational modification, A276 and C277 are the likely candidates that determine the outcome of the p53 DBD-DNA interaction. Other than speculation, no experiments have directly addressed the degree of involvement of A276 or C277 in apoptosis or cell cycle arrest. Since the thiol (SH) group of cysteine is known to form disulfide bonds¹⁰ and cysteine itself participates in numerous post-translational modifications¹¹, we decided to refrain from mutating C277 due to its critical functions and narrowed our focus to A276 of p53.

Systematic analysis of the DNA-binding sites of p53 with all known promoters showed that p53 has a higher binding affinity for gene promoters involved in cell cycle, DNA repair than apoptotic promoters¹². Based on this observation, the crystal structure of p53 DBD bound to two sets of palindromic DNA half-sites with either cytosine (C9) or thymine (T9) located at the ninth position was solved to understand these specific interactions². Sequential inspection of these promoter regions revealed that most apoptotic gene promoters and the 3'-promoter site of the cell cycle gene, p21, have cytosine (C) in their ninth position of the target decameric sequence whereas the rest of the promoters such as DNA repair and other cell cycle genes have thymine (T). In wild-type p53, the methyl side chain (carbon 8) of A276 contacts the methyl group (carbon 7) of T9 via a hydrophobic interaction as depicted in Figure 2A. However, in the presence of C9, A276 moves away from the hydrophilic site and is not involved in interacting with DNA (Figure 2B).

If alanine is substituted with a hydrophilic residue such as serine, this may now interact with C9, whereas substitution with a more hydrophobic residue such as phenylalanine may interact better with T9. Modeling of A276S showed that the hydroxyl (OH) group of serine can orient itself at the center of C9, G8 (guanine at the 8th position) and their phosphate groups (Figure 2D). The nitrogen 4 of C9 may make contact with the OH group of A276S via direct or water-mediated hydrogen bonding. Moreover, A276S can also form non-specific hydrogen bonds with both phosphate groups of DNA and a specific hydrogen bond with the nitrogen 7 of G8. With T9, A276S faces the opposite side of the DNA half-site as illustrated in Figure 2C. Similar to wild-type as predicted, A276F mutation prefers a hydrophobic interaction with T9 (Figure 2E) but no interaction with C9 at the DNA half-site (Figures 2F). Therefore, we hypothesized that alteration from a hydrophobic to a hydrophilic interaction between 276th amino acid of p53 and C9 of the DNA half-site should induce higher apoptosis yet maintain cell cycle arrest.

Materials and Methods

Molecular Modeling

Snapshots were taken with molecular visualization software PyMOL (The PyMOL Molecular Graphics System Version 1.5.0.4 Schrödinger, LLC.) initiated from the 2ATA and 2ACO² PDB structure. Using the display option, A276 bound to the DNA half-site was visualized as stick model and color-coded to distinguish it from the rest of the structure. The DNA half-site contains either an A/T or G/C base pair at the ninth position; PyMOL Mutagenesis Wizard was used to generate A276S and A276F mutations associated with this DNA half-site. Both backbone-dependent and backbone-independent rotamers were simulated at 0.3 fps to obtain the least-hindered conformation of the mutated residues. In addition to sterility, the conformation with the closest possible distance from the target nucleotide unit (A/T or G/C) was selected to generate the representative images in Figure 2.

Construction of Plasmids and Mutagenesis

EGFP-p53 (pEGFP-p53) plasmid was constructed as previously described¹³. E-p53ΔTD (amino acids 1-322) plasmid was truncated at BspEI and KpnI restriction sites. Using site-directed mutagenesis, EGFP-p53 plasmid was mutated to create A276-variant p53 with the following mutagenic primers: A276S, forward 5'-CTTTGAGGTGCGTGTTTGTTCCTGTCCTGGGAGAGACCGGCG-3' and A276S, backward 5'-CGCCGGTCTCTCCAGGACAGGAACAAACACGCACCTCAAAG-3', A276G, forward 5'-TTTGAGGTGCGTGTTTGTGGCTGTCCTGGGAGAGACCGGC-3' and A276G, backward 5'-GCCGGTCTCTCCAGGACAGCCACAAACACGCACCTCAAA-3', A276F, forward 5'-CTTTGAGGTGCGTGTTTGTTCCTGTCCTGGGAGAGACCGGCG-3' and A276F, backward 5'-CGCCGGTCTCTCCAGGACAGAAACAAACACGCACCTCAAAG-3', A276R, forward 5'-CTTTGAGGTGCGTGTTTGTGCTGTCCTGGGAGAGACCGGCG-3' and A276R, backward 5'-CGCCGGTCTCTCCAGGACAGCGACAAACACGCACCTCAAAG-3', A276Q, forward 5'-CTTTGAGGTGCGTGTTTGTGAATGTCCTGGGAGAGACCGGCG-3' and A276Q, backward 5'-CGCCGGTCTCTCCAGGACATTACAAACACGCACCTCAAAG-3', A276N, forward 5'-CTTTGAGGTGCGTGTTTGTAACTGTCCTGGGAGAGACCGGCG-3', and A276N, backward 5'-CGCCGGTCTCTCCAGGACAGTTACAAACACGCACCTCAAAG-3'. All plasmids are subcloned into the same vector, EGFP, which contains a strong, constitutively active CMV promoter.

Cell Lines and Transient Transfections

Human cervical carcinoma HeLa cells (ATCC, Manassas, VA) were grown as monolayers in RPMI (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin-L-glutamate (Invitrogen), and 0.1% gentamicin (Invitrogen). This was used as the growth medium for human ductal breast epithelial cells T47D (ATCC) was further supplemented with 4mg/L of insulin (Sigma, St. Louis, MO). All cells were maintained in a 5% CO₂ incubator at 37°C. 3 × 10⁵ cells for T47D and 7.5 × 10⁴ cells for HeLa were plated in 6-well plates for most assays (Greiner Bio-One, Monroe, NC) or in 2-well live cell chambers for fluorescence microscopy (Nalge Nunc, Rochester, NY), unless stated otherwise. Twenty-four hours after seeding, the cells were transfected with 3.5μg of DNA per well using Lipofectamine® 2000 (Invitrogen) per manufacturer's instructions as previously¹³.

Luciferase Assay

All constructs (3.5µg of DNA) were transfected in T47D cells with either p53-Luc Cis-Reporter (Agilent), pBV-Luc PUMA reporter (a generous gift from Dr. Vogelstein, Addgene plasmid 16591)¹⁴, pGL3-Luc GADD45WT reporter (a generous gift from Michael Greenberg, Addgene plasmid 8356)¹⁵, pGL3-Basic PERP reporter (a generous gift from Dr. T. Jacks, Addgene 14810)¹⁶ or pBluescript II KS (+) + luc p21WAF1/CIP reporter (a generous gift from Dr. Vogelstein, Addgene plasmid 16451)¹⁷ each encoding firefly luciferase. 0.35µg of pRL-SV40 plasmid encoding renilla luciferase (a generous gift from Dr. Phillip Moos, University of Utah) was cotransfected with every reporter plasmid to normalize transfection efficiency. The Dual-Glo Luciferase Assay System (Promega, Madison, WI) was used as previously¹³ to determine the activity of firefly and renilla luciferase following the manufacturer's recommendations. PlateLumino luminometer (Strattec Biomedical Systems, Birkenfeld, Germany) was used to detect luciferase activity of transfected cells 24 h post-transfection. Firefly luciferase values were normalized for renilla luciferase. EGFP transfected cells served as a negative control and EGFP-p53 transfected cells served as a positive control. The assay was repeated in three independent experiments (n=3).

Fluorescence Microscopy and DNA Segmentation

Forty-eight hours following transfection in 2-well live cell chambers, 0.3 OL of H33342 (nuclear dye, Invitrogen) was added to T47D cells expressing the construct of interest and incubated for 30 min at 37°C. Cells were analyzed with an inverted fluorescence microscope (Olympus IX701F, Scientific Instrument Co., Sunnyvale, CA, USA) as previously described¹⁸, with high-quality narrow band GFP filter (excitation HQ480/20 nm, emission HQ510/20 nm, beam splitter Q4951p, Chroma Technology Corp., Brattleboro, VT, USA) equipped with an F-view Monochrome CCD camera. The EGFP fluorescence field (blinded to nuclei) was viewed and were imaged with a 60X oil immersion objective as previously described¹⁹. The nuclei of at least 100 transfected cells (EGFP fluorescence) per sample were classified as either healthy (round or kidney shaped) or segmented (punctate)^{20, 21}. To quantitate the percentage of transfected cells with segmented DNA, the fluorescent image was analyzed using AnalySIS® software (Soft Imaging System, Lakewood, CO) and averaged from three separate experiments (n=3).

Cell Proliferation and Western Blot

Cell proliferation analysis was carried out as previously described²². In short, for the proliferation analysis, 24 h, 48 h, and 72 h following transfection of T47D cells, trypan blue (Invitrogen) exclusion was performed to determine the number of viable cells. For Western blot analysis, 24 h following transfection of T47D cells, EGFP-positive cells were sorted using the FACS Aria-II (BD-BioSciences). 3×10^5 cells were pelleted and resuspended in 200 µL lysis buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 1% protease inhibitor). Standard western blotting procedures were followed using primary antibodies to detect p21WAF1/CIP1 and actin as a loading control. The primary antibodies anti-p21 (ab16767, Abcam, Cambridge, MA) and anti-actin (ab3280, Abcam) were detected with anti-mouse HRP-conjugated secondary antibody (ab6814, Abcam) before the addition of SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Waltham, MA). Bands were detected with a FluorChem FC2 imager (Alpha Innotech, Santa Clara, CA).

7-AAD Assay

Hela and T47D cells were stained with 7-aminoactinomycin D (7-AAD, Invitrogen) according to the manufacturer's instructions. HeLa cells were tested after 24 h and T47D cells were tested after 48 h and 72 h, respectively following transfection. The samples were

analyzed using the FACSCanto-II (BD-BioSciences, University of Utah Core facility) with FACSDiva software. EGFP and 7-AAD were excited with a 488 nm laser. Sample analysis was based on EGFP positive cells, and all samples were gated at same EGFP intensity levels. All constructs were tested in three independent experiments (n=3).

Cell Cycle Arrest

For cell cycle arrest analysis, 1×10^6 T47D cells were transfected as described above (Cell Lines and Transient Transfection). Forty-eight hours post-transfection, adherent cells were collected, washed in PBS (Invitrogen), and fixed in 70% cold ethanol at -20°C overnight. Fixed cells were centrifuged at 2000 rpm for 5 min and washed three times with PBS. Cells were then suspended in stain solution containing 10 % v/v of 0.5 mg/mL propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO), 5% v/v PBS, 84 % v/v of 4mM citrate buffer (Sigma-Aldrich), 0.62 % v/v RNase A (Sigma-Aldrich), 1% v/v Triton X-100 (Sigma-Aldrich), and 3% w/v of PEG 6000 (Fluka, St. Louis, MO) for 20 min at 37°C in the dark. An equal volume of salt solution containing 5% of PI, 1% of Triton X-100, 89% v/v of 0.4M NaCl (Thermo Fisher Scientific Inc, Pittsburgh, PA), and 3% m/v of PEG 6000 was added to the stained cells and incubated at 4°C for 1 h²³ in the dark as well. Cells were gated at same intensity level of EGFP. EGFP and PI were excited at 488 nm and detected at 507 nm and 670 nm, respectively. Samples were repeated in three distinct experiments (n=3).

Statistical Analysis

Data were represented as mean $\pm$ standard deviation and analyzed using one-way ANOVA with Tukey's post-hoc test. Statistical differences between the same construct at different time points was compared via Two-way ANOVA, Bonferroni test using GraphPad Prism software. A p value <0.05 was considered significant

Results

Selection of amino acids for A276 mutation

In order to experimentally verify the importance of A276, wild-type p53 was mutated at this position to the select amino acids with side chains varying in nature, size, charge, and hydrophobicity (Table 1). Even though the side chains of both alanine (A) and phenylalanine (F) are hydrophobic, they differ in size and degree of hydrophobicity. The smallest amino acid, glycine (G), lacks a side chain and therefore can elucidate the necessity of a side chain at 276th position. Compared to only three possible hydrogen bonds with serine(S), the amide group of asparagine (N) can form five potential hydrogen bonds simultaneously. Despite both being neutral, serine and asparagine differ in size and hydrophilicity. Glutamic acid (E) and arginine (R) are negatively and positively charged residues, respectively that possess the ability to make, if any, salt bridges with neighboring charged residues. Overall, this selected set of mutations approximately all the side chain properties of standard amino acids.

A276S shows elevated transcriptional activity of a promoter containing C9 at the DNA half-site

To screen and compare the transcriptional activity, we subjected all p53 mutants (listed in Table 1) to the p53-cis reporter system. Upon p53 recognition to its repeated synthetic promoter consensus (TGGCCTGGACTTGCCTGG)₁₄²⁵, firefly luciferase is expressed in proportion to the binding of p53 to the reporter. As depicted in Figure 3, all constructs failed to display higher transcriptional activity compared to the negative control (EGFP) with the exception of A276S and wild type p53. As expected from the presence of C9 in the p53 recognition sequence (see Table 2, top row), A276S showed significantly higher

transcriptional activity than wild-type p53 (E-p53), validating our proposed modeling in Figure 2D. Since the majority of p53 induced apoptotic gene promoters also have C9 at the target decameric sequences¹², we decided to pursue with A276S, a small hydrophilic residue, as a promising candidate to induce higher apoptosis compared to wild-type p53. Conversely, A276F with a bulky hydrophobic residue serves as an inactivating mutation for our experiments.

A276S transactivates apoptotic and cell cycle genes

To investigate whether the preferred C9 binding of A276S translates into elevated transactivation, we compared transcriptional activity from several different p53 target gene promoters in T47D cells. These selected genes, PUMA, PERP, p21^{WAF1/CIP1}, and GADD45 have defined decameric promoter sequences, and are known to be involved in diverse p53-mediated functions as listed in Table 2. Like the p53-binding reporter above, the promoters of these genes are fused with luciferase. The resultant luminescence can therefore indicate the transcriptional activity of target gene promoters upon binding to wild-type or mutant p53.

PUMA and PERP are apoptotic genes targeted by p53. Once expressed, PUMA translocates to the mitochondria and interacts with anti-apoptotic Bcl-XL and Bcl-2 proteins to mediate rapid apoptosis via the intrinsic pathway¹⁴. PERP (PMP-2) is a membrane-bound protein that acts as a cell death receptor and is speculated to be important for the extrinsic apoptotic pathway¹⁶. A276S displayed significantly higher PUMA (Figure 4A) and PERP (Figure 4B) gene promoter transcription than wild-type p53. As previously (Figure 3), the negative control (EGFP) and A276F showed minimal transcriptional activity. Enhanced transcription of these gene promoters suggests that A276S can better induce the mediators of both p53-dependent intrinsic and extrinsic apoptotic pathways than wild-type p53.

p21^{WAF1/CIP1}, a downstream target of p53, inhibits cyclin-dependent kinases (CDK) leading to G1/G0 cell cycle arrest¹⁷. p21^{WAF1/CIP1} also modulates apoptosis^{29, 30}. As shown in Figure 4C, A276S showed increased p21 promoter transcription compared to wild-type p53. Since p21^{WAF1/CIP1} involves in both apoptosis and cell cycle arrest, we decided to investigate the protein expression level of this established target of p53. Protein expression is the most direct and robust method to validate the transcription and translation of any gene. In this case, the overexpression of p21^{WAF1/CIP1} target protein will be an indication of enhanced binding of A276S to the C9 of the gene promoter. A276S displayed higher p21^{WAF1/CIP1} protein expression in a western blot compared to wild-type p53 (Supplementary Figure 1). Parallel to the negative control (EGFP), A276F failed to induce p21 gene promoter (Figure 4C). This result shows that A276S not only binds specifically to an apoptotic gene promoter but also successful to elevate its protein expression which should lead to higher apoptosis compared to wild-type p53. In addition, the same protein expression trend can be observed when compared to the p21^{WAF1/CIP1} transcription assay (Compare Supplementary Figure 1 to Figure 4C), validating the reliability of this reporter assay for the expression of other genes.

GADD45 stimulates DNA repair excision upon p53 transcription³¹. However, the promoter of GADD45 gene contains a thymine (Table 2, bottom row) at position 9 instead of cytosine. As expected, A276S showed lower transcription of GADD45 gene promoter compared to wild type p53 (Figure 4D).

A276S maintains cell cycle arrest

To further evaluate the CDK inhibitory effect of increased p21^{WAF1/CIP1} expression, we examined the cell cycle arrest potential of each construct. E-p53 Δ TD lacks the

tetramerization domain (TD); thus, it resides as an inactive monomeric form in the nucleus. Since our cell cycle analysis method detects the nuclei, we used monomeric E-p53 Δ TD as a negative control to be able to gate for EGFP transfected cells. Wild-type p53 significantly arrests cells in G0/G1 phase than p53 Δ TD ($p < 0.01$) (Figure 5, compare 5A to 5B) as expected. Although the p21^{WAF1/CIP1} expression profile of A276S was higher than wild-type p53, it demonstrated similar cell cycle arrest (Figure 5C). It can be speculated that after a certain threshold level, any further expression of p21^{WAF1/CIP1} may not contribute to increased cell cycle arrest. A276F also succeeded to arrest cells better than the negative control (Figure 5D). Other than p21^{WAF1/CIP1}, A276F may bind to several p53 target genes such as B99, cyclin-G, BDS, GML¹² or via an unknown pathway to induce the observed cell cycle arrest.

A276S inhibits cell proliferation

Next, the improved transcriptional activity of A276S was analyzed for its effect in T47D cells. As increased expressions of apoptotic genes and cell cycle arrest at G0/G1 phase are expected to stagnate growth, cell proliferation was monitored by cell counts after 48 and 72 hours following transfection using trypan blue exclusion. As seen in Figure 6, 48 hours post-transfection, A276S (2nd bar) and wild-type p53 (1st bar) similarly reduced cell proliferation compared to A276F (3rd bar). However, at 72 hours, A276S (5th bar) caused a significant reduction in cell proliferation compared to wild-type p53 (4th bar). Not surprisingly, A276F showed significant reduction in cell viability after 72 hours in agreement to the cell cycle arrest observation. This provides the evidence (albeit indirect) that over time A276S effectively employs cell death and cell cycle arrest together to inhibit cell proliferation.

A276S educes hallmark of apoptosis

Analysis of cell morphology is a widely used method to visualize the induction of apoptosis¹⁹. Among these hallmarks, nuclear segmentation can be quantified to measure apoptosis of transfected cells. Cells morphologies of different constructs were photographed as depicted in Figure 7A. The averaged segmented nuclei were quantified and graphed as shown in Figure 7B. Congruent to the previous experiments, A276S demonstrated higher percentage of segmented cells versus wild-type p53. A276F behaved similar to EGFP (Figure 7B). With elevated expression of PUMA, PERP, and p21^{WAF1/CIP1}, indeed A276S induces apoptosis better than wild-type p53.

A276S executes higher cell death than wild-type p53

To confirm the end-point of apoptosis, we conducted a flow cytometric experiment with 7-AAD which measures late apoptosis or necrosis. The percentage of 7-AAD positive T47D cells was measured 48 and 72 hours post transfection as depicted in Figure 8A. At both time points, A276S (3rd bar and 7th bar) demonstrated significantly higher late apoptosis than wild-type p53 (2nd bar and 6th bar). A276F (4th bar) and EGFP (1st bar) failed to achieve significant cell death. Moreover, each construct was compared at different time points. A276S induces higher cell death at 72 hours (7th bar) than at 48 hours (3rd bar) following transfection. Wild-type p53 remained statistically indistinguishable at both time points. Since T47D cells have mutated and mislocalized endogenous p53, constructs were also tested in HeLa cells with endogenous wild-type p53. In HeLa cells, the 7-AAD assay was conducted at 24 hours due to their higher sensitivity to apoptosis. As observed in Figure 8B, A276S successfully displayed the same apoptotic trend, regardless of endogenous p53 status. EGFP and A276F (1st and 4th bar, respectively, Figure 8B) behaved as expected and failed to induce apoptosis. This result, together with apoptotic promoter transcription and nuclear segmentation, establishes that A276S induces higher apoptosis than wild-type p53.

Discussion

Analysis of crystallographic structure of p53 bound to DNA revealed several direct DNA-binding residues. Among these, L120, A276, and C277 are reported as the three critical residues for selecting target promoters. A previous study showed that A276 in p53 specifically interacts with the 9th position of the target DNA decameric sequence². The methyl group of A276 was found to interact with the methyl group of thymine via a hydrophobic interaction. However, in the presence of a cytosine at position 9 of a target decameric sequence, the methyl group of A276 did not interact with the hydrophilic N7 site. Interestingly, another key study reported that a number of apoptosis and cell cycle arrest gene promoters have a cytosine at position 9, while for most DNA repair gene promoters, thymine is found instead¹². Since our goal is to improve p53 as a cancer therapeutic, we were interested in modifying p53 to improve its apoptotic function. From this, we hypothesized that substitution of a hydrophilic residue (A276S) would selectively increase binding to C9 of apoptotic gene promoters. Therefore, the A276S mutant should induce higher apoptosis than wild-type p53, yet maintain cell cycle arrest.

Molecular modeling was performed to visualize the possible interactions between wild-type p53, A276S, or A276F and the p53-target decameric sequence containing either a C9 or a T9. As opposed to wild-type p53, A276S favored binding to the decameric sequence at position 9 containing a cytosine instead of a thymine. We speculated that the hydroxyl (OH) group of serine might be advantageous over the methyl group of alanine to form a hydrogen bond with the nitrogen of C9. In addition, the lone pair of oxygen might make water-mediated hydrogen bonds with the phosphate backbone of the target DNA. With significant steric hindrance, mainly because of size, A276F preferred binding to the decameric sequence with a thymine at position 9 rather than a cytosine.

A276S was selected as a promising candidate to bind better to promoters containing C9. Failure of other selected amino acids (F, G, S, N, E, and R) signified the importance of the 276th position for promoter transcription, identifying A276 as a hot spot for p53. A276F was chosen as a negative control, in addition to EGFP, due to modeling and initial lack of p53 reporter gene assay.

The benefit of enhanced binding of A276S to C9 was tested in T47D cells, by measuring the transcription of selected gene promoters using a luciferase-based assay. Four gene promoters were tested: PUMA, PERP and p21^{WAF1/CIP1}. PUMA expression leads to the intrinsic apoptotic pathway, PERP follows the extrinsic apoptotic pathway, p21^{WAF1/CIP1} modulates cell cycle arrest as well as apoptosis, and GADD45 repairs DNA damage. A276S demonstrated significantly higher transcription of PUMA, PERP, and p21^{WAF1/CIP1} gene promoters and lower transcription of GADD45 promoter compared to wild-type p53. As expected, the negative controls, A276F and EGFP failed to transactivate these gene promoters. Up-regulation of these genes suggested the possible mechanisms by which A276S could induce higher apoptosis than wild-type p53 and maintained cell cycle arrest.

The outcome of increased p21^{WAF1/CIP1} expression (a CDK inhibitor) was examined in cell cycle regulation. All three constructs, wild-type p53, A276S, and surprisingly A276F displayed similar cell cycle arrest. As anticipated, the negative control (monomeric p53 Δ TD) apprehended less G0/G1 DNA content than all other constructs. It is thought that overexpression of p21^{WAF1/CIP1} after a threshold point may mediate cell death rather than cell cycle arrest. In addition, A276F might bind to other cell cycle gene promoters¹² or undergone an unknown pathway that lead to the observed cell cycle arrest.

A276S also inhibited cell proliferation over an extended time period. Cell counts normalized to EGFP showed that A276S maintained the same number of viable cells over a 72 hour

period. Even though initially the same as A276S, wild-type p53 treated cells were more viable over time. This indirectly demonstrated that A276S can efficiently implement apoptosis and cell cycle arrest versus wild-type p53. In agreement to the observed cell cycle experiment, A276F also reduced cell proliferation over time to some extent (although not as potently as A276S or wild-type p53).

Following the elevated transactivation of PUMA and PERP, A276S illustrated higher induction of apoptosis than wild-type p53, as measured through 7-AAD staining, and morphological signs of apoptosis in T47D cells (with mutated and mislocalized endogenous p53). A276S caused higher cell deaths in HeLa cell lines (with unmutated endogenous p53) as well. These studies provide evidence that A276S can also execute higher apoptosis in a different cell line, regardless of the endogenous p53 status.

In this paper, we demonstrated that our rationally-designed A276S-mutant p53 can up-regulate apoptotic target gene promoters and thereby cause higher cell death than wild-type p53. We have addressed A276 as a hot spot of p53. Interestingly, A276D mutation has been reported in patient³². We also identified the select amino acid properties required to fit and favorably interact with the 276th position. Even though a few improvised p53-variants have been discovered mostly in terms of protein stability^{33–35}, here we report a new p53 mutant that induces higher apoptosis by directly binding to a specific DNA sequence.

p53 is a top candidate for gene therapy for cancer. Of the three main functions of p53, apoptosis, cell cycle arrest, and DNA repair, we focused on improving the apoptotic function of p53. Our proposed p53 mutant, A276S, can specifically bind to and transactivate the promoters of selected apoptotic genes but not DNA repair or other off target genes. In terms of therapeutic efficacy, A276S induced higher apoptosis than wild-type p53 yet maintained cell cycle arrest. These studies indicate the utility of using A276S mutant p53 for cancer therapy. Although the biological activity of A276S was explored in this paper, further evidence on biochemical characterization is required to determine its biophysical properties such as protein stability. Further studies of A276S with other p53 promoters and in other cell line could expand the “enhanced death star” properties of p53, and prove its utility as a potent anticancer agent, with greater efficacy than wild-type p53.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic diagram of p53 structure and possible pathways upon oxidative stress/DNA damage. Arrows indicate direct DNA-binding residues of p53.

Figure 2.

Stick model of p53-A276 interacting with DNA. Light gray color indicates wild type (WT) p53 residues surrounding A276 position; green indicates carbon; blue indicates nitrogen; red indicates oxygen; white indicates hydrogen; and orange represents phosphorus. Residues circled in black indicate hydrophobic sites and squares indicate hydrophilic sites. C9 represents cytosine located at the 9th position of p53 and T9 is thymine at the 9th position. (A) A276:T9. The hydrophobic interaction between carbon 8 of A276 (circled) and carbon 7 of T9 (circled) is depicted with the orange dashed line. (B) A276:C9. Replacement of T9 with C9 nucleotide shows loss of interaction between A276 and target DNA. (C) A276S:T9; substitution of alanine with serine. The OH group of serine moves away from the hydrophobic carbon 7 of T9 (circled). (D) A276S:C9. The square shows possible hydrogen bonding or water-mediated interaction between the OH-group of A276S and Nitrogen 4 of C9 as illustrated with purple dashed lines. In addition, A276S may form hydrogen bonds with both phosphate groups of G8 and C9. (E) A276F:T9; substitution of alanine with phenylalanine. The benzene ring of phenylalanine serves as a hydrophobic pocket for the circled methyl group of T9. (F) A276F:C9. The aromatic ring of A276F is repulsed from the hydrophilic environment of G8 and C9.

Figure 3.

Luciferase assay: Relative luminescence illustrates the ability of mutated p53 constructs to activate the p53-cis reporter in T47D cells. Mean values were analyzed using one-way ANOVA with Tukey's post test (** $p < 0.001$). Error bars represent standard deviations from independent experiments ($n=3$).

Figure 4.

Transcription of (A) PUMA, (B) PERP, (C) p21WAF1/CIP1 and (D) GADD45 promoters in luciferase system. Mean values are analyzed with one-way ANOVA, Tukey's post test (*p<0.05, **p<0.01 ***p<0.001). Error bars represents standard deviations from independent experiments.

Figure 5.

Cell cycle arrest: All constructs were assessed for DNA content 48 hour post-transfection in T47D cells. E-p53 Δ TD represents monomeric p53 as a negative control instead of EGFP. The values reported are averaged DNA content $\pm$ s.d. of transfected cells in G0/G1 phase. (A) p53 Δ TD (B) E-p53 (wild-type) (C) A276S and (D) A276F. Statistical analysis was performed using one-way ANOVA with Tukey's post test.

Figure 6.

Viable T47D cells after 48 and 72 hours following transfection. The number of proliferating cells for each construct was normalized to the number of EGFP transfected viable cells (negative control). Values are reported as mean $\pm$ s.d; one-way ANOVA, Tukey's post test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 7.

Nuclear segmentation of T47D cells.(A) Representative image of T47D cells 48 hours post transfection with either EGFP(top row), E-p53 (middle row), or A276S (bottom row).

Complete arrows pointed to healthy nuclei of transfected cells and broken arrows pointed to segmented nuclei. (B) Quantification of segmented nuclei. Percentages of transfected cells were determined from independent experiments. Values reported as mean $\pm$ s.d (* $p < 0.05$); one-way ANOVA, Tukey's post test.

Figure 8.

Late apoptosis/necrosis analysis. 7-AAD assay (A) in T47D cells after 48 and 72 hours post-transfection and (B) in HeLa cells after 24 hours post transfection. Averaged values are reported with standard deviations from separate experiments; one-way ANOVA, Tukey's post test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$); two-way ANOVA, Bonferroni test (# $p < 0.05$).

Table 1Properties of mutated amino acids ^a

Mutation	Amino acid characteristics	Side-Chain Charge (pH 7.4)	Size (kDa) ³⁶	Hydrophobic index ²⁴
A276F	Hydrophobic	Neutral	165.19	100
A276(wt)	Hydrophobic	Neutral	89.10	44
A276G	Neutral	N/A	75.07	0
A276S	Hydrophilic	Neutral	105.09	-5
A276N	Hydrophilic	Neutral	132.12	-25
A276E	Hydrophilic	Negative	147.13	-60
A276R	Hydrophilic	Positive	174.10	-75

^a A276 denotes alanine at 276th position of p53 followed by the one letter code of mutated amino acid residue. Positive values represent hydrophobic side chains, negative values represent hydrophilic side chains, and 0 represents no side-chain. The charges on the side chains are positive, neutral or negative.

Table 2

The p53-consensus for target decameric sequences ^b

		Potential p53-DNA binding site									
		1	2	3	4	5	6	7	8	9	10
Promoter consensus	Function	R	R	R	C	W	W	G	Y	Y	Y
p53 recognition	Binding consensus	G	G	A	C	T	T	G	C	C	T
PUMA	Apoptosis	G	G	G	C	T	A	G	g	C	C
PERP	Apoptosis	G	c	G	C	T	A	G	T	C	C
p21	Cell cycle/Apoptosis	G	G	G	C	A	T	G	T	C	T
GADD45	DNA repair	A	A	G	C	A	T	G	C	T	g

^bThe top row represents decameric sequence (R=A or G; W= A or T; Y= C or T) ¹². The 9th positions of promoters are either C or T. Lowercase of this table represent exceptions to the consensus. References: p53 recognition²⁵; PUMA¹⁴; p21 WAF1/CIP1²⁶; PERP²⁷; GADD45²⁸.